

Comparative Structure-Activity Analysis of Insect Kinin Core Analogs on Recombinant Kinin Receptors From Southern Cattle Tick *Boophilus microplus* (Acari: Ixodidae) and Mosquito *Aedes aegypti* (Diptera: Culicidae)

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The systematic analysis of structure-activity relationships of insect kinins on two heterologous receptor-expressing systems is described. Previously, kinin receptors from the southern cattle tick, *Boophilus microplus* (Canestrini) [Holmes et al., *Insect Mol Biol* 9:457–465 (2000); Holmes et al., *Insect Mol Biol* 12:27–38 (2003)], and the dengue vector, the mosquito *Aedes aegypti* (L.) [Pietrantonio et al., *Insect Mol Biol* 14:55–67 (2005)], were functionally and stably expressed in CHO-K1 cells. In order to determine which kinin residues are critical for the peptide-receptor interaction, kinin core analogs were synthesized as an Ala-replacement series of the peptide FFSWGa and tested by a calcium bioluminescence plate assay. The amino acids Phe¹ and Trp⁴ were essential for activity of the insect kinins in both receptors. It was confirmed that the pentapeptide kinin core is the minimum sequence required for activity and that the C-terminal amide is also essential. In contrast to the tick receptor, a large increase in efficacy is observed in the mosquito receptor when the C-terminal pentapeptide is N-terminally extended to a hexapeptide. The aminoisobutyric acid (Aib)-containing analog, FF[Aib]WGa, was as active as superagonist FFSWGa on the mosquito receptor in contrast to the tick receptor where it was statistically more active than FFSWGa by an order of magnitude. This restricted conformation Aib analog provides information on the conformation associated with the interaction of the insect kinins with these two receptors. Furthermore, the analog FF[Aib]WGa has been previously shown to resist degradation by the peptidases ACE and neprilysin and represents an important lead in the development of biostable insect kinin analogs that ticks and mosquitoes cannot readily deactivate. *Arch Insect Biochem Physiol* 620:128–140, 2006. Published 2006 Wiley-Liss, Inc.[†]

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INTRODUCTION

The insect kinins (leucokinin-like peptide family or myokinins) are multi-functional neuropep-

tides that have been found in several invertebrate and arthropod groups (Nässel, 1996; Torfs et al., 1999; Coast et al., 2002a; Gade, 2004; Riehle et al., 2002). Eight closely related myotropic neu-

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ropeptides, designated leucokinin I–VIII, were first isolated from the cockroach, *Leucophaea maderae*, by their stimulatory actions on hindgut contraction (Holman et al., 1986, 1990a,b; Nachman and Holman, 1991). Shortly after their discovery, leucokinins from the cockroach were shown to have diuretic activity on isolated Malpighian tubules of the yellow fever mosquito *Aedes aegypti* (Hayes et al., 1989). Cockroach and endogenous *Aedes* kinins also depolarize the transepithelial voltage in isolated Malpighian tubules (Hayes et al., 1989; Veenstra et al., 1997; Pietrantonio et al., 2000). Subsequently, of the three endogenous *Aedes* kinins (Aedae-K), the Aedae-K-1 and -3 were shown to have diuretic activity in vitro (Veenstra et al., 1997). The in vivo studies have shown that both diuresin (from *Culex salinarius*) and the three *Aedes* kinins when injected in *A. aegypti* females, increase urine production in a dose-dependent manner (Cady and Hagedorn, 1999).

Leucokinins activate G protein-coupled receptors (GPCRs) that transduce the hormonal signal through heterotrimeric guanine nucleotide-binding proteins (G proteins) through an increased production of IP₃, causing the release of calcium from intracellular stores through the IP₃ receptor cascade (Radford et al., 2002; Cady and Hagedorn, 1999). In the mammalian Chinese hamster ovary (CHO-K1) cell expression system, kinin receptors from *Aedes aegypti* and the tick *Boophilus microplus* also elevate intracellular calcium in response to kinins or kinin analogs (Pietrantonio et al., 2005; Holmes et al., 2003).

Most leucokinins are characterized by the C-terminal pentapeptide Phe-Xaa-Ser-Trp-Gly-NH₂ where X is Phe, His, Ser, or Tyr (Nachman and Holman, 1991; Holman et al., 1999; Torfs et al., 1999). Myotropic and diuretic assays of tissues in vitro show that the full biological activity of the insect kinins resides in the C-terminal pentapeptide, which is the active core (Nachman and Holman, 1991; Nachman et al., 2003), with the exception of the housefly Malpighian tubule fluid secretion assay (Coast et al., 2002b) where the C-terminal pentapeptide core is less potent by several orders of magnitude. Diuretic and myotropic

activity in these assays is completely lost when the C-terminal amide of the insect kinins is replaced with a negatively charged acid moiety (Nachman et al., 1995). Within the core pentapeptide, the aromatic residues Phe¹ and Trp⁴ are the most important for activity whereas a wide range of variability is tolerated at position 2, from acidic to basic residues and from hydrophilic to hydrophobic (Nachman and Holman, 1991; Nachman et al., 1993). A plausible receptor interaction model proposes that the aromatic side chains of Phe¹ and Trp⁴ are oriented towards the same region and interact with the receptor. Conversely, the side chain of residue 2 lies on the opposite face pointing away from the receptor surface, which explains why this position is more tolerant to changes (Nachman et al., 2002b).

Further studies with α -amino-isobutyric acid (Aib) residue at the third position in the core pentapeptide showed that the analog is as active as the natural neuropeptide (Nachman et al., 1997). Nuclear magnetic resonance and molecular modeling studies on the insect kinin analog FF[Aib]WG_a revealed that it can exist as two different β -turns comprising residues Phe¹ to Trp⁴ or Phe² to Gly⁵ (Moyna et al., 1999). Further NMR studies with insect kinin analogs incorporating either the tetrazole or 4-aminopyroglutamate, moieties that mimic one turn over the other, indicate a predominant population of a β -turn involving the Phe¹ to Trp⁴ region (Nachman et al., 2002b, 2004).

Myokinin receptors from the southern cattle tick, *Boophilus microplus* (Holmes et al., 2000, 2003), and the dengue vector, the mosquito *Aedes aegypti* (Pietrantonio et al., 2005), were previously stably and functionally expressed in CHO-K1 cells. Here for the first time we present a systematic analysis of structure-activity relationships of the C-terminal pentapeptide core region of the insect kinins on these two heterologous receptor-expressing systems. In order to determine which myokinin residues are critical for the peptide-receptor interaction, kinin core analogs were synthesized as an Ala-replacement series or "alanine scan" and were tested by a calcium bioluminescence assay previously described (Pietrantonio et al., 2005). We also

tried to determine the minimal size of an active myokinin core and the effect of the C-terminal OH group on the myokinin receptor response. In addition, we evaluated a restricted conformation analog of the insect kinins, previously shown to have potent activity in a diuretic assay (Nachman et al., 1997), that incorporates the sterically-bulky residue aminoisobutyric acid (Aib) and sheds light on conformations associated with the interaction of the insect kinins with the two receptors.

MATERIALS AND METHODS

Peptide Analogs

The analogs were synthesized on an ABI 433A Peptide Synthesizer using a modified FastMoc0.25 procedure as well as manually by the solid-phase method, using the Fmoc-strategy starting from Rink Amide resin (Novabiochem, 0.53 mM/g). The Fmoc protecting group was removed by 20% piperidine in DMF. A fourfold excess of the respective Fmoc-amino acids was activated in situ using HBTU (1eq.)/HOBT (1eq.) in NMP (automated synthesis) or DCM (manual synthesis) and coupling reactions were base catalyzed with DIPEA (4 equivalents). The amino acid side chain protecting group was tBu for Ser. The analogs were cleaved from the resin with side-chain deprotection by treatment with TFA:H₂O:TIS (95.5:2.5:2.5 v/v/v) for 1.5 h. The total volume of the TFA filtrate was reduced to about 1 ml and then precipitated with cold diethyl ether. The solvents were evaporated under reduced pressure and the resulting materials dissolved in water and lyophilized.

The analogs were purified on a Waters C₁₈ Sep Pak cartridge and a Delta-Pak C₁₈ reverse-phase column (8 × 100 mm, 15 µm particle size, 100 Å pore size) on a Waters 510 HPLC controlled by a Millennium 2010 chromatography manager system (Waters, Milford, MA) with detection at 214 nm at ambient temperature. Solvent A = 0.1% aqueous trifluoroacetic acid (TFA); Solvent B = 80% aqueous acetonitrile containing 0.1% TFA. Conditions: Initial solvent consisting of 20% B was followed by the Waters linear program to 100% B over 40

min; flow rate, 2 ml/min. Delta-Pak C-18 retention times: FFSWAa, 13.5 min; FFSAGa, 4.75 min; FFAWGa, 10.5 min; FASWGa, 6.0 min; AFSWGa, 7.25 min; FF[Aib]WGa, 12.0 min; FFSWa, 7.8 min; FSWGa, 10.5 min; FFSWG-OH, 6.0 min; FFSWGa, 9.0 min; and FFFSWGa, 12.25 min. The analogs were further purified on a Waters Protein Pak I125 column (7.8 × 300 mm) (Milligen Corp., Milford, MA). Conditions: Flow rate: 2.0 ml/min; isocratic with Solvent = 80% acetonitrile made to 0.01% TFA; WatPro retention times: FFSWAa, 6.25 min; FFSAGa, 7.5 min; FFAWGa, 6.0 min; FASWGa, 7.5 min; AFSWGa, 7.5 min; FF[Aib]WGa, 6.0 min; FFSWa, 6.0 min; FSWGa, 8.75 min; FFSWG-OH, 6.0 min; FFSWGa, 6.0 min; and FFFSWGa, 6.0 min. These HPLC conditions have been described in detail elsewhere (Nachman et al., 2004). Amino acid analysis was carried out under previously reported conditions (Nachman et al., 2004) and used to quantify the peptides and to confirm identity, leading to the following analyses: FFSWAa: A[1.0], F[2.0], S[1.0]; FFSAGa: A[1.0], G[1.2], F[2.0], S[1.0]; FFAWGa: A[1.0], G[1.0], F[2.0]; FASWGa: A[1.0], G[0.8], F[1.0], S[0.9]; AFSWGa: A[0.9], G[0.8], F[1.0], S[0.9]; FF[Aib]WGa: G[0.8], F[2.0]; FFSWa: F[2.0], S[0.9]; FSWGa: G[0.7], F[1.0], S[0.9]; FFSWG-OH: G[1.3], F[2.0], S[1.3]; FFSWGa: G[1.1], F[2.0], S[0.9]; and FFFSWGa: G[1.0], F[3.0], S[1.0]. The identity of the analogs was confirmed via MALDI-MS on a Kratos Kompact Probe MALDI-MS machine (Kratos Analytical, Ltd., Manchester, UK) with the presence of the following molecular ions (MH⁺): FFSWAa, 656.3[MH⁺]; FFSAGa, 527.0[MH⁺]; FFAWGa, 625.9[MH⁺]; FASWGa, 565.9[MH⁺]; AFSWGa, 566.4[MH⁺]; FF[Aib]WGa, 639.8[MH⁺]; FFSWa, 585.3[MH⁺]; FSWGa, 494.5[MH⁺]; FFSWG-OH, 643.1[MH⁺]; FFSWGa, 642.4[MH⁺]; and FFFSWGa, 789.9[MH⁺].

Cell Lines

Receptor cloning, transfection, and selection of single clonal cell lines expressing the kinin (leukokinin-like peptide) receptors from the Southern cattle tick, *B. microplus* (AF228521), and the yellow fever mosquito, *A. aegypti* (AY596453), was re-

ported previously (Holmes et al., 2000, 2003; Pietrantonio et al., 2005). The CHO-K1 cell lines expressing, respectively, the tick receptor, BmLK3 (Holmes et al., 2003), and the *Aedes* kinin receptor, E10 (Pietrantonio et al., 2005), were maintained in F-12K medium (Invitrogen, La Jolla, CA) supplemented with 10% fetal bovine serum (EquiTech Bio, Kerrville, TX) with 400 µg/ml GENETICIN® at 37°C and 5% CO₂.

Analysis of Myokinin Peptide Analog Activity by a Ca²⁺ Bioluminescence Plate Assay

The functional analysis of peptides on stably transformed CHO-K1 cells expressing myokinin receptors was by intracellular calcium measurements as described (Pietrantonio et al., 2005). The assay uses aequorin, a photoprotein isolated from luminescent jellyfish (*Aequorea victoria*), and other marine organisms. Aequorin consists of a 189-amino acid polypeptide in a complex that includes a reactive group, coelenterazine, and oxygen. Upon addition of calcium ions, the photoprotein undergoes a conformational change. Filling of the calcium-binding sites on this protein results in the oxidation of bound coelenterazine using the protein-bound oxygen to form excited coelenteramide. When excited coelenteramide relaxes to a ground state, it emits light at 469 nm (Mithofer and Mazars, 2002).

Briefly, the aequorin plasmid mtAEQ/pcDNA1 (a kind gift from Drs. C.J.P. Grimmlikhuijzen and Michael Williamson, University of Copenhagen, Denmark) was grown in *Escherichia coli* cells MC1061/P3 (Invitrogen) and was purified with a Qiaprep spin miniprep kit (Qiagen Inc., Chatsworth, CA). Transient transfection with this plasmid was as described by Staubli et al. (2002). For this, the cells expressing the myokinin receptors were grown in F12K media containing 10% fetal bovine serum and 400 µg/ml GENETICIN® to about 90% confluency in T-25 flasks at 37°C and 5% CO₂. Cells were trypsinized and 2 × 10⁵ cells in 2 ml of media were seeded in each well of 6-well tissue culture plates. For a typical assay, 2–3 wells were sufficient. Cells were allowed to grow

for 24 h in the incubator and typically they were 60% confluent at this time. The media was removed and replaced with OPTI-MEM media (Gibco, Invitrogen Co.). For transfection of cells in each well, 96 µl of OPTI-MEM media was mixed with 4 µl of the transfection reagent Eugene 6 (Roche Biochemicals) in a microfuge tube. The mixture was incubated for 5 min at room temperature after which 1 µg of aequorin/pcDNA1 plasmid DNA in 10 mM Tris buffer, pH 8.5, without EDTA was added and incubated for another 15–20 min at room temperature. This mixture (typically 105–106 µl) was added dropwise to each well with gentle manual shaking; plates were incubated for 4–6 h and then the media was changed to F12K media containing 10% fetal bovine serum without antibiotic. After 24 h, cells were trypsinized and transferred to 96-well, white, thin bottom micro-titer plates (Costar 3610, Cambridge, MA) at a density of 40,000 cells/100 µl per well and incubated for 24 h after which they reached a confluency of 80%, optimal for performing the bioluminescence assay. To reconstitute the aequorin complex, cells were incubated in 90 µl/well of calcium-free DMEM media (GIBCO, Invitrogen Co.) containing 5 µM coelenterazine (Molecular Probes, Eugene, OR) for 3 h (Stables et al., 1997) in the dark at 37°C and 5% CO₂. Cells were then challenged with different concentrations of peptide analogs in a volume of 10 µl (10×) solubilized in calcium-free DMEM media. In all the experiments, FFFSWGa, a potent hexapeptide agonist for both mosquito and tick kinin receptors, was used as a positive control (Holmes et al., 2003; Pietrantonio et al., 2005). The assay was performed using the NOVOstar (BMG Labtechnologies) plate reader in bioluminescence mode at room temperature. Light emission (469 nm) was recorded every 2 s over a period of 50 s per well. In order to compare the myokinin receptors' response to various peptides and to analyze the time course for their response to different peptides, two types of histograms were constructed using the GraphPad Software 4.0 (GraphPad Software Inc., San Diego, CA). In one type of histogram, the maximum bioluminescence response at 1 µM peptide concentration was compared among

all the different peptides studied. In the second type of histogram, the bioluminescence response measured every 2 s after the addition of 1 μ M peptide was plotted against time in seconds. Analysis of activity for each peptide was repeated at least three times with two replicates each. Concentration-response curves were obtained by nonlinear regression curve fit analysis (sigmoidal dose-response equation with variable slope) using Prism software 4.0. Maximal responses from six individual replicates at each concentration were used for calculations of the EC_{50} 's.

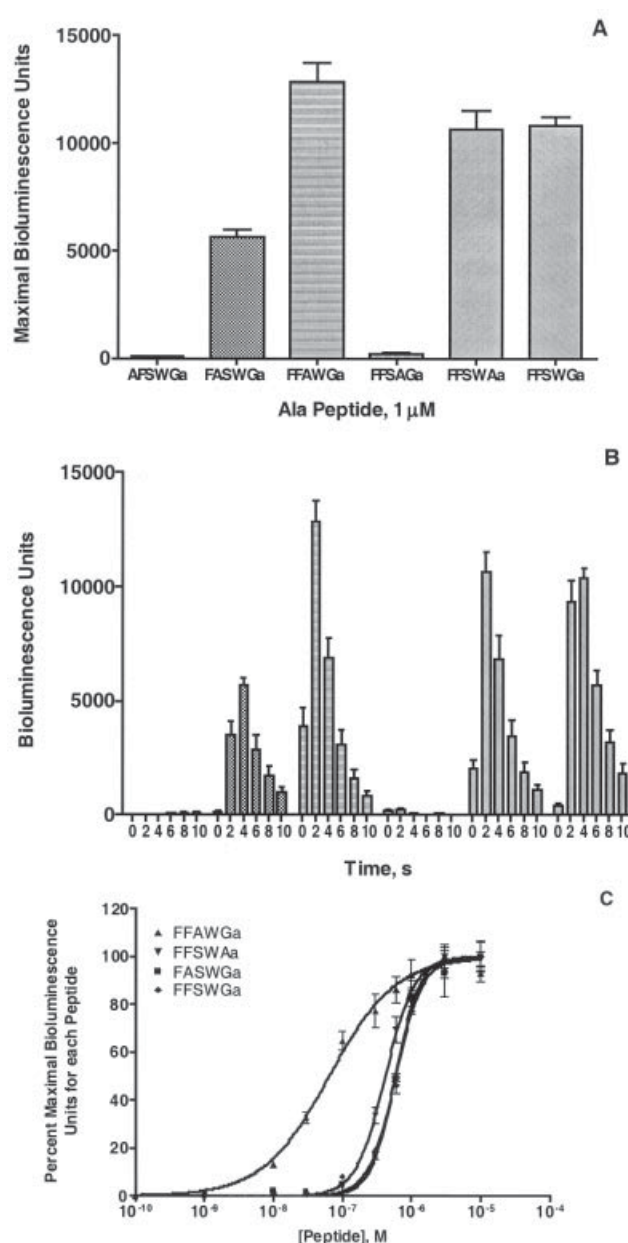
RESULTS

Effect of Ala Substitution on the Activity of the Insect Kinin Core

In order to investigate the role of individual residues of the insect kinin core peptide FFSWG_a, the kinin core and five different Ala substituted analogs of this peptide (Ala substitution at each of the five positions) were synthesized. The analogs were tested on tick and mosquito kinin receptors

stably expressed in CHO-K1 cells using a functional calcium bioluminescence assay. First, analogs were screened at 1 μ M concentration (Figs. 1A,B) to define which analogs would be further studied for the determination of their effective concentration fifty (EC_{50}). On the tick receptor (BmLK3 cell line), the analog FFAWG_a was found to elicit the greatest response at 1 μ M followed by FFSWA_a and FFSWG_a (equal response), and, lastly, with apparent lesser activity, FASWG_a. Analogs AFSWG_a and FFSAG_a failed to show any response on the tick

Fig. 1. Alanine replacement series scan of a kinin agonist core region (FFSWG_a) on the tick myokinin receptor by a calcium bioluminescence plate assay. **A**: Maximal bioluminescence response of five different Ala analogs at 1- μ M concentration. **B**: Time course of bioluminescence response of the same analogs at 1 μ M concentration; several concentrations from 10 μ M to 1 nM were tested but only one is shown in Figures 1–6. Bioluminescence was measured every 2 s for 50 s. (For clarity, the response shown is for only 10 s.) **C**: Estimation of the effective concentration fifty (EC_{50}) of the five Ala peptide analogs. Bioluminescence units measured for the different peptide concentrations were expressed as a percentage of the maximal bioluminescence response observed among all concentrations tested for each peptide. Analog FFAWG_a was statistically more potent than the others. Identical bar fillings were used for the same peptide in A and B, and the vertical lines on the bars represent standard errors of independent experiments, from a maximum of six to a minimum of three, each consisting of measurements from two wells. Statistical analysis and graphs were created with the GraphPad Prism 4.0 software. Refer to Table 1 for EC_{50} values.



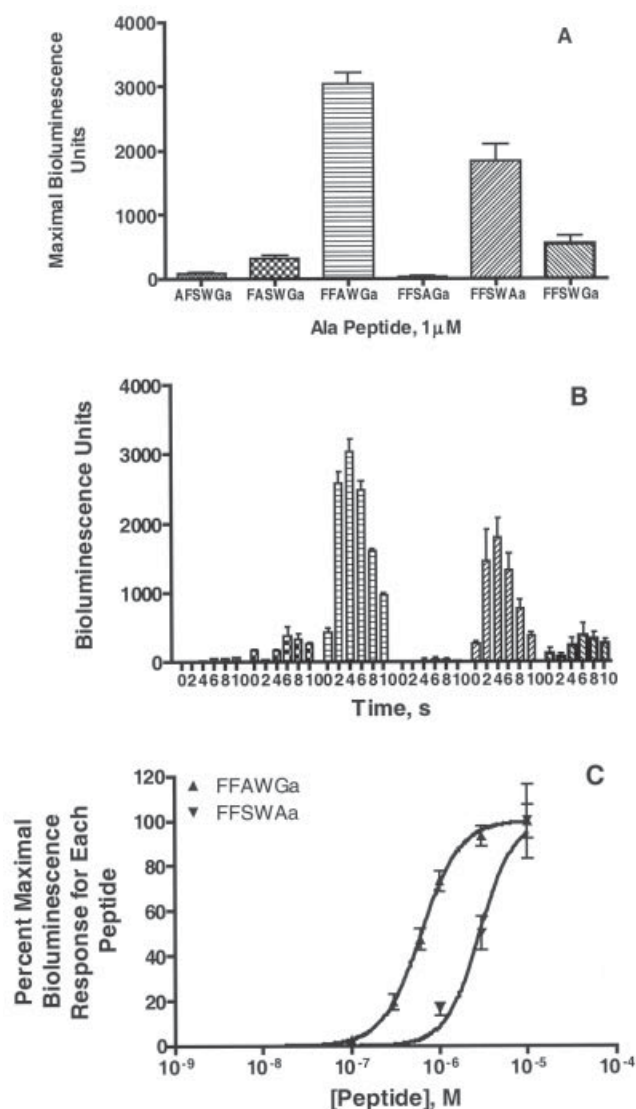


Fig. 2. Alanine replacement series scan of a kinin agonist core region (FFSWGa) on the mosquito kinin receptor by a calcium bioluminescence plate assay. **A:** Maximal bioluminescence response of five different Ala analogs at 1- μ M concentration. **B:** Time course of bioluminescence response of the same analogs at 1- μ M concentration. Bioluminescence was measured every 2 s for 50 s. (For clarity, the response shown is for only 10 s.) **C:** Estimation of EC₅₀ of different peptide analogs. Bioluminescence units measured for the different peptide concentrations were expressed as a percentage of the maximal bioluminescence response observed among all concentrations tested for each peptide. Identical bar fillings were used for the same peptide in A and B, and the vertical lines on the bars represents standard errors of independent experiments, from a maximum of six to a minimum of three, each consisting of measurements from two wells. Refer to Table 1 for EC₅₀ values.

receptor (Fig. 1A). Bioluminescence was measured for 50 s in all cases but only measurements for the first 10 s are shown here (Figs. 1B and 2B). The bioluminescence response declines quickly in tick receptor expressing cells, which is probably caused by receptor desensitization. Determination of the EC₅₀ revealed that the order of potency was FFAWGa > FFSWAa = FASWGa = FFSWGa, based on the respective EC₅₀ values of FFAWGa, 64 nM; FFSWAa, 417 nM; FASWGa, 586 nM; FFSWGa, 590 nM (Fig. 1C). Analog FFAWGa was the most potent and its dose-response curve was statistically different ($P < 0.05$) from those of other analogs, while dose-response curves for analogs FFSWGa, FFSWAa, and FASWGa were not statistically different ($P = 0.9$) among themselves (Fig. 1C).

On the mosquito receptor (E10 cell line), the most potent analog was also FFAWGa followed in potency by FFSWAa as observed for the tick receptor (Fig. 2A). As observed on the tick BmLK3 cell line, analogs FFSAGa and AFSWGa did not show any response on the mosquito receptor. As only analogs FFAWGa and FFSWAa showed significant response at 1- μ M concentrations, the EC₅₀ of these two peptides was calculated. The order of potency of Ala analogs on the mosquito receptor was FFAWGa > FFSWAa based on their EC₅₀ values of FFAWGa, 621 nM; FFSWAa, 2.8 μ M, which were statistically significantly different ($P < 0.05$). For both the tick and mosquito receptors, the dose-response curves for FFAWGa and FFSWAa showed a similar difference of about one order of magnitude (Fig. 2C).

Contrary to the response on the tick receptor, analogs FASWGa and FFSWGa showed very little response even at 1 μ M on the mosquito receptor.

Minimal Size and Terminal OH Group

Two analogs FSWGa and FFSWa were designed to confirm the minimal size of kinin analog required for activity and one analog having the OH group at its C terminus, FFSWG-OH, was designed to demonstrate the importance of the C-terminal amide. All of the analogs failed to elicit any response on the tick and mosquito receptors (Figs. 3 and 4). Even peptides tested at 10 μ M concentration did not show any effect in both receptors.

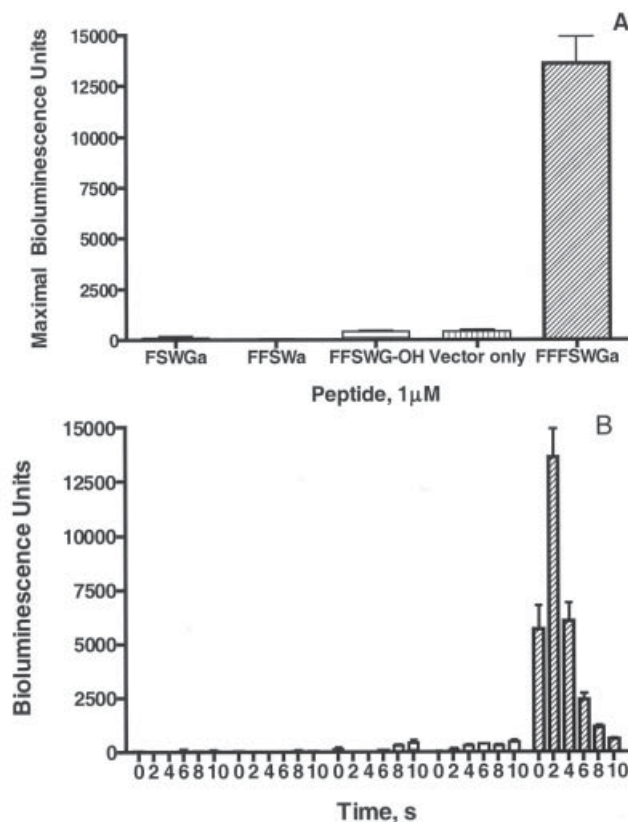


Fig. 3. Effect of kinin agonist truncation and C-terminal amide replacement by the OH group on peptide activity on the tick myokinin receptor. **A:** Maximal bioluminescence response of two truncated analogs, an acid and superagonist FFFSWGa at 1 μ M concentration. **B:** Time course of bioluminescence response of the same analogs at 1 μ M concentration. Bioluminescence units measured for the different peptide concentrations were expressed as a percentage of the maximal bioluminescence response observed among all concentrations tested for each peptide. Identical bar fillings were used for the same peptide in A and B, and the vertical lines on the bars represents standard errors of independent experiments, from a maximum of six to a minimum of three, each consisting of measurements from two wells.

These results show that the minimum fragment required for the activity is a pentapeptide with a C terminal amide.

Restricted Conformation Analog

The Aib-containing analog, FF[Aib]WGa, exhibited a response comparable to that of the hexa-

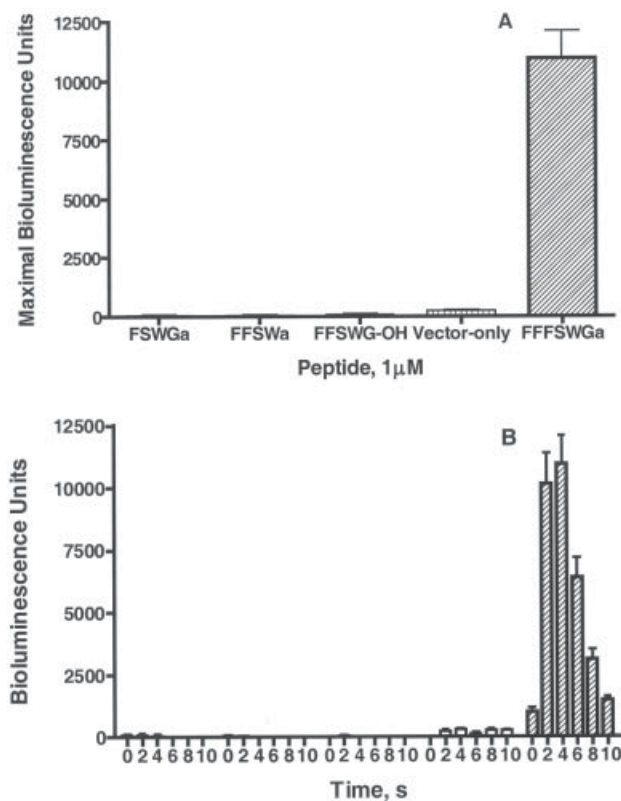


Fig. 4. Effect of kinin agonist truncation and C-terminal amide replacement by the OH group on peptide activity on the mosquito kinin receptor. **A:** Maximal bioluminescence response of two truncated analogs, an acid and superagonist FFFSWGa, at 1 μ M concentration. **B:** Time course of bioluminescence response of the same analogs at 1 μ M concentration. Bioluminescence units measured for the different peptide concentrations were expressed as a percentage of the maximal bioluminescence response observed among all concentrations tested for each peptide. Identical bar fillings were used for the same peptide in A and B, and the vertical lines on the bars represents standard errors of independent experiments, from a maximum of six to a minimum of three, each consisting of measurements from two wells.

peptide FFFSWGa at 1 μ M concentration, both on the tick (Fig. 5A) and on the mosquito receptors (Fig. 6A). The hexapeptide FFFSWGa had been previously found to be most potent among different peptides in activating the tick myokinin receptor in a fluorescence calcium assay using the same cell line (Holmes et al., 2003). The rank order of potencies was FFFSWSa = FFFSWGa > FFSWGa >

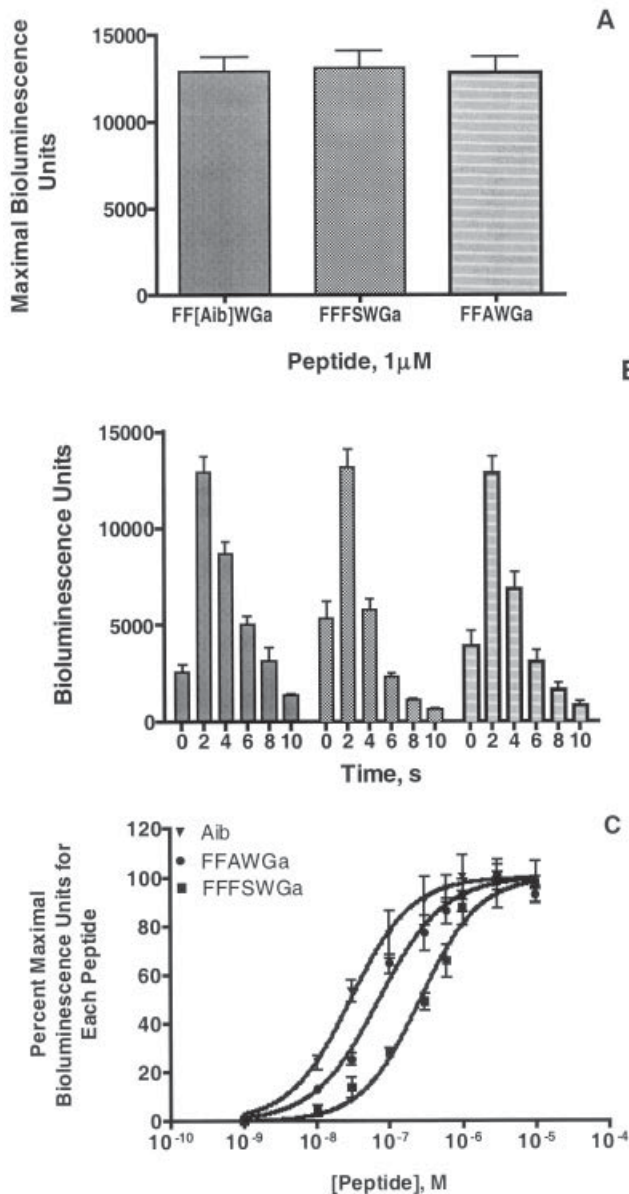


Fig. 5. Activity comparison of the restricted conformation analog FF[Aib]WGa with the synthetic superagonist FFFSWGa and Ala analog FFAWGa on the tick myokinin receptor. A: Maximal bioluminescence response to the FF[Aib]WGa, FFFSWGa, and FFAWGa analogs of insect kinin peptides at 1-μM concentration. B: Time course of bioluminescence response of the same analogs at 1-μM concentration. C: Estimation of EC₅₀ of FF[Aib]WGa, FFFSWGa, and FFAWGa. The y-axis in the concentration-response curves was obtained from bioluminescence units expressed as a percentage of the maximal response observed for each peptide. Analog FF[Aib]WGa was statistically significantly more potent than FFFSWGa and FFAWGa; $P < 0.05$. Bioluminescence units measured for the different peptide concentrations were expressed as a percentage of the maximal bioluminescence response observed among all concentrations tested for each peptide. Identical bar fillings were used for the same peptide in A and B, and the vertical lines on the bars represents standard errors of independent experiments, from a maximum of six to a minimum of three, each consisting of measurements from two wells.

quito receptor with the estimated EC₅₀ for FF[Aib]WGa being 445 nM and for FFFSWGa 562 nM, which were not statistically different (Fig. 6C).

In summary, the rank order of potency of analogs for the tick receptor was FF[Aib]WGa > FFAWGa > FFFSWGa (Fig. 5C and Table 1), with

TABLE 1. Estimated Potencies (EC₅₀) and Maximal Bioluminescence Response of All the Peptides Tested on Tick (BmLK3) and Mosquito (E10) Receptor Transfected Cell Lines*

Peptides	Tick receptor (BmLK3 cell line)		Mosquito receptor (E10 cell line)	
	EC ₅₀ (nM)	Maximal bioluminescence response at 1 μM	EC ₅₀ (nM)	Maximal bioluminescence response at 1 μM
FFSWGa	—	—	—	—
FASWGa	586	5,600	N.D.	400
FFAWGa	64	12,800	621	3,050
FFSAWGa	—	—	—	—
FFSWGa	417	10,600	2,800	1,830
FFSWGa	590	10,800	N.D.	525
FSWGa	—	—	—	—
FFSWa	—	—	—	—
FFSWG-OH	—	—	—	—
FFFSWGa	259	13,000	562	10,000
FF[Aib]WGa	29	12,700	445	9,300

*The EC₅₀ estimates the concentration required to induce a half-maximal response. I: Inactive if bioluminescence response is less than 300 units (level of vector-only transfected cells). A: The position where the respective residue in the peptide FFFSWGa has been replaced by alanine.

FYSWGa > muscakinin > lymnokinin (Holmes et al., 2003). In the current study, the hexapeptide was also found to elicit the greatest response at 1 μM for both tick and mosquito receptor since it produced the highest number of bioluminescence units among all the peptides studied (see Figs. 1 and 2 vs. Figs. 5 and 6, respectively, and Table 1). The EC₅₀ values were calculated and FF[Aib]WGa was more potent on the tick receptor with an EC₅₀ of 29 nM, an order of magnitude lower than the EC₅₀ of 259 nM for FFFSWGa (Fig. 5C). In contrast, both peptides were equipotent on the mos-

the three EC_{50} being statistically different. In contrast, all three peptides were equipotent for the mosquito kinin receptor (Fig. 6C, Table 1).

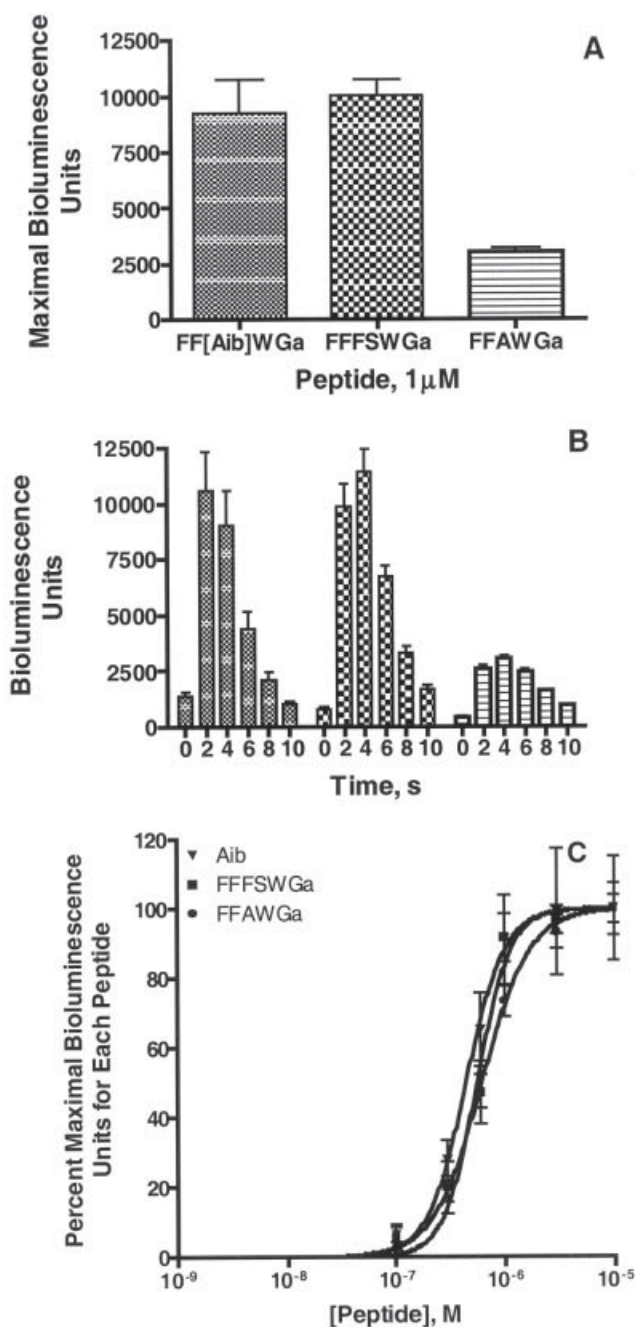
DISCUSSION

Previous studies (Coast et al., 1990, 2002a,b; Nachman et al., 1990; Nachman and Holman, 1991) have shown that the C-terminal insect kinin pentapeptide is the minimal active fragment that retains biological activity in cricket and housefly diuretic assays as well as a cockroach hindgut myotropic assay. In the current study, we have shown that the C-terminal insect kinin pentapeptide fragment FFSWGa retains activity on insect kinin receptors from the Southern cattle fever tick *Boophilus microplus* and the disease vector mosquito *Aedes aegypti* expressed in a heterologous system using a calcium bioluminescence plate assay. By contrast, the analogs FSWGa and FFSWa, representing truncations of the pentapeptide at the N-terminus and C-terminus, respectively, fail to show a response even up to 10 μ M (Figs. 3 and 4) (Table 1). This demonstrates that the C-terminal pentapeptide represents the minimal core required to elicit a response from the tick and mosquito receptors. As with the diuretic and myotropic assays

cited above, the C-terminal amide is critical for interaction of the insect kinins with both the tick and mosquito receptors as the analog FFSWG-OH fails to elicit a significant response in either case (Figs. 3 and 4) (Table 1).

Evaluation of a series of Ala-substituted analogs, an Ala scan, of the C-terminal pentapeptide FFSWGa demonstrates that two of the analogs, AFSWGa and FFSAGa, were completely inactive on both tick and

Fig. 6. Activity comparison of the restricted conformation analog FF[Aib]WGa with the synthetic superagonist FFSWGa and Ala analog FFAWGa on the mosquito kinin receptor. **A:** Maximal bioluminescence response to FF[Aib]WGa, FFSWGa, and FFAWGa analogs of insect kinin peptides at 1- μ M concentration. **B:** Time course of bioluminescence response of the same analogs at 1- μ M concentration. **C:** Estimation of EC_{50} of FF[Aib]WGa, FFSWGa, and FFAWGa. Bioluminescence units measured for the different peptide concentrations were expressed as a percentage of the maximal bioluminescence response observed among all concentrations tested for each peptide. The curves for analogs FF[Aib]WGa, FFSWGa, and FFAWGa were not statistically significantly different ($P = 0.8$). Identical bar fillings were used for the same peptide in A and B, and the vertical lines on the bars represents standard errors of independent experiments, from a maximum of six to a minimum of three, each consisting of measurements from two wells.



mosquito receptors at a concentration of 1 μ M (Figs. 1 and 2). This demonstrates the requirement of the aromatic side chains of Phe¹ and Trp⁴ for the activity of the insect kinin pentapeptide core, also noted in earlier studies performed using diuretic and myotropic assays (Nachman and Holman, 1991; Nachman et al., 1993; Roberts et al., 1995). This is consistent with a plausible receptor interaction model (Nachman et al., 1990, 2002b; Roberts et al., 1995) in which the insect kinin pentapeptide approaches the receptor leading with the critical Phe¹/Trp⁴ aromatic surface, leaving the residue at variable position 2 pointing away from the binding site. The two receptors also demonstrate similar responses to the analog FFAWGa, which proves statistically more active than other pentapeptide analogs, including the parent peptide (Figs. 1 and 2). The sidechain of Ser is not a critical component of the interaction with these receptor sites. This rise in activity observed for FFAWGa in the mosquito receptor is perhaps a consequence of a natural in vivo functional interaction with a native insect kinin that contains an Ala at that position (*Aedes* kinin-2: NPFHAWGa). However, this peptide was not re-tested in this study but it is second in potency after *Aedes* kinin 3 for the mosquito kinin receptor (Pietrantonio et al., 2005).

For the tick receptor, the EC₅₀ for FFAWGa is more potent than that of FFSWGa by an order of magnitude. Although the sequence(s) of tick kinin(s) are unknown at this time, it is possible that at least one of them contains an Ala at position three. In addition, the analog FASWGa is less active in both receptors than other members of the Ala-substitution series, demonstrating a preference for an aromatic residue at core position 2. This is not surprising in the case of the mosquito receptor, given that *Aedes* kinins-1, -2, and -3 contain aromatic residues at this position (Tyr, His, and Tyr, respectively). Again, the sequence(s) of kinin(s) native to the tick are not known.

However, at least one difference in the responses to other Ala-substitution analogs is evident between the tick and mosquito receptors. The tick receptor is not sensitive to replacement of the C-terminal Gly position with Ala (analog FFSWAa),

whereas this change leads to a response that is statistically higher on the mosquito receptor (Figs. 1 and 2). In addition, it should be noted that the C-terminal pentapeptide FFSWGa appears to be a better ligand for the tick receptor than it is for the mosquito. Nevertheless, a previous study has shown that FFSWGa elicits a change in the transepithelial voltage of *Aedes* Malpighian tubules at an EC₅₀ of 3×10^{-10} M (Pietrantonio et al., 2000). The difference in potency observed between these different assays is, in part, due to the fact that the receptors in this study are expressed in mammalian cells. The mosquito receptor, however, responds very strongly to the addition of a Phe at the N-terminus of the pentapeptide core. The hexapeptide FFFSWGa elicits a significantly stronger response at 1 μ M than any of the other active pentapeptide analogs, with the magnitude of this increase ranging from a factor of 3 to 20 (Figs. 2 and 6, Table 1). Clearly, efficacy is enhanced on going from an insect kinin C-terminal pentapeptide to a hexapeptide in the mosquito receptor. The greater response observed for FFFSWGa over FFSWGa is consistent with a study of the change in the transepithelial voltage on *Aedes* Malpighian tubules where the rank order of potency was found to be FFFSWGa > FFSWGa > FYSWGa (Pietrantonio et al., 2000). In contrast, the maximal response of the hexapeptide FFFSWGa is only slightly higher than that of the other active pentapeptide analogs in the tick receptor (Fig. 1, Table 1). In the tick receptor, efficacy does not improve markedly on going from an insect kinin C-terminal pentapeptide to a hexapeptide fragment.

Despite the steric bulk in the backbone of the Aib-containing analog FF[Aib]WGa, it nevertheless elicits a very strong calcium bioluminescence response in both tick and mosquito receptors. This is in agreement with the potent activities of Aib-containing analogs observed in a cricket Malpighian tubule fluid secretion assay, an in vivo housefly diuretic assay, and a cockroach hindgut myotropic assay (Nachman et al., 1997, 2002a). In the mosquito receptor, it is statistically equipotent with the superagonist FFFSWGa, whereas in the tick receptor, it is an order of magnitude more

potent than this same superagonist (Figs. 5 and 6). For the tick receptor, the FF[Aib]WGa pentapeptide analog was also more potent than the FF Δ WGa analog. The Aib-containing analog is structurally more related to this Ala analog FF Δ WGa than to any of the other peptides tested. This structural similarity is perhaps responsible for its equipotency to the FF Δ WGa peptide for the mosquito kinin receptor (Fig. 6, Table 1). Therefore, it is the most potent peptide analog yet observed for the tick receptor, and matches the activity of the most potent peptide in the mosquito receptor. The steric bulk of the Aib residue also restricts the number of conformations available to the backbone of this analog, and provides some insight into the conformation adopted by the insect kinins at the two receptors. A previous solution conformation study using both NMR spectroscopic data and molecular dynamics calculations concludes that the analog adopts only two major turn conformations. These consist of a turn over residues Phe¹ through Trp⁴, comprising 60% of the population, and another over residues Phe² through Gly⁵, comprising the remaining 40% (Moyna et al., 1999; Nachman et al., 1990, 2002b; Roberts et al., 1995). Subsequent studies on the Malpighian tubule fluid secretion activity of insect kinin analogs that incorporate components that specifically mimic the Phe¹ to Trp⁴ turn, such as the tetrazole and 4-aminopyroglutamate motifs (Nachman et al., 2002b, 2004), demonstrated that this turn is the active conformation in the cricket diuretic bioassay. The potent activity observed for FF[Aib]WGa in both the tick and mosquito insect kinin receptors may be a consequence of its ability to mimic the Phe¹ to Trp⁴ β -turn. Evaluation of additional peptidomimetic, restricted conformation analogs will be undertaken in the future to further define the conformation critical to the interaction of the insect kinins with the tick and mosquito receptors.

In conclusion, structure-activity relationships for the interaction of insect kinins with receptors from the tick and mosquito gleaned from these experiments provide important information relevant to the development of biostable, bioavailable analogs with the potential to disrupt the diuretic, myo-

tropic, and/or digestive processes these neuropeptides regulate. Indeed, the potent activity of FF[Aib]WGa is an interesting observation given that the steric bulk of the Aib residue confers resistance to degradation by peptidases such as ACE and neprilysin that attack the native insect kinins at the peptide bond between the Ser³ and Trp⁴ core residues (Nachman et al., 2002a). The Aib analog's enhanced biostability can serve as a tool for insect neuroendocrinologists in their quest to understand the function of the insect kinins in the mosquito and particularly the tick, for which their role remains unknown. This peptidase-resistant analog represents an important lead in the development of biostable insect kinin analogs that cannot be deactivated by ticks and mosquitoes, important pests of man and livestock, and may aid in the development of new neuropeptide-based strategies to control them.

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LITERATURE CITED

- Cady C, Hagedorn HH. 1999. The effect of putative diuretic factors on in vivo urine production in the mosquito, *Aedes aegypti*. *J Insect Physiol* 45:317–325.
- Coast GM, Orchard I, Phillips JE, Schooley DA. 2002a. Insect diuretic and antidiuretic hormones. *Adv Insect Physiol* 29:279–41.
- Coast GM, Zabrocki J, Nachman RJ. 2002b. Diuretic and myotropic activities of N-terminal truncated analogs of *Musca domestica* kinin neuropeptide. *Peptides* 23:701–708.

- Coast GM, Holman GM, Nachman RJ. 1990. The diuretic activity of a series of cephalomyotropic neuropeptides, the achetakinins, on isolated Malpighian tubules of the house cricket, *Acheta domesticus*. *J Insect Physiol* 36:481–488.
- Gade G. 2004. Regulation of intermediary metabolism and water balance of insects by neuropeptides. *Annu Rev Entomol* 49:93–113.
- Hayes TK, Pannabecker TL, Hinckley DJ, Holman GM, Nachman RJ, Petzel DH, Beyenbach KW. 1989. Leucokinins, a new family of ion transport stimulators and inhibitors in insect Malpighian tubules. *Life Sci* 44:1259–66.
- Holman GM, Cook BJ, Nachman RJ. 1986. Primary structure and synthesis of a blocked myotropic neuropeptide isolated from the cockroach, *Leucophaea maderae*. *Comp Biochem Physiol C* 85:219–24.
- Holman GM, Nachman RJ, Wright MS. 1990a. Comparative aspects of insect myotropic peptides. In: Eppl A, Scanes CG, Stetson MH, editors. *Progress in comparative endocrinology*. New York: Wiley-Liss. p 35–39.
- Holman GM, Nachman RJ, Wright MS. 1990b. Insect neuropeptides. *Annu Rev Entomol* 35:201–17.
- Holman GM, Nachman RJ, Coast GM. 1999. Isolation, characterization and biological activity of a diuretic myokinin neuropeptide from the housefly, *Musca domestica*. *Peptides* 20:1–10.
- Holmes SP, He H, Chen AC, Ivie GW, Pietrantonio PV. 2000. Cloning and transcriptional expression of a leucokinin-like peptide receptor from the southern cattle tick, *Boophilus microplus* (Acari: Ixodidae). *Insect Mol Biol* 9:457–465.
- Holmes SP, Barhoumi R, Nachman RJ, Pietrantonio PV. 2003. Functional analysis of a G protein-coupled receptor from the southern cattle tick *Boophilus microplus* (Acari: Ixodidae) identifies it as the first arthropod myokinin receptor. *Insect Mol Biol* 12:27–38.
- Mithofer A, Mazars C. 2002. Aequorin-based measurements of intracellular Ca²⁺-signatures in plant cells. *Biol Proced Online* 4:105–118.
- Moyna G, Williams HJ, Nachman RJ, Scott AI. 1999. Conformation in solution and dynamics of a structurally constrained linear insect kinin pentapeptide analogue. *Biopolymers* 49:403–413.
- Nachman RJ, Holman GM. 1991. Myotropic insect neuropeptide families from the cockroach *Leucophaea maderae*: Structure-activity relationships. In: Menn JJ, Masler EP, editors. *Insect neuropeptides: Chemistry, biology, and action*. Washington, DC: American Chemical Society. p 194–214.
- Nachman RJ, Roberts VA, Holman GM, Tainer JA. 1990. Consensus chemistry and conformation of an insect neuropeptide family analogous to the tachykinins. In: Eppl A, Scanes CG, Stetson MH, editors. *Progress in comparative endocrinology*, Vol. 342. New York: Wiley-Liss, Inc. p 60–66.
- Nachman RJ, Roberts VA, Holman GM, Haddon WF. 1993. Leads for insect neuropeptide mimetic development. *Arch Insect Biochem Physiol* 22:181–197.
- Nachman RJ, Coast GM, Holman GM, Beier RC. 1995. Diuretic activity of C-terminal group analogues of the insect kinins in *Acheta domesticus*. *Peptides* 16:809–813.
- Nachman RJ, Isaac RE, Coast GM, Holman GM. 1997. Aib-containing analogues of the insect kinin neuropeptide family demonstrate resistance to an insect angiotensin-converting enzyme and potent diuretic activity. *Peptides* 18:53–57.
- Nachman RJ, Strey A, Isaac E, Pryor N, Lopez JD, Deng JG, Coast GM. 2002a. Enhanced in vivo activity of peptidase-resistant analogs of the insect kinin neuropeptide family. *Peptides* 23:735–745.
- Nachman RJ, Zabrocki J, Olczak J, Williams HJ, Moyna G, Ian Scott A, Coast GM. 2002b. cis-peptide bond mimetic tetrazole analogs of the insect kinins identify the active conformation. *Peptides* 23:709–716.
- Nachman RJ, Coast GM, Douat C, Fehrentz J, Kaczmarek K, Zabrocki J, Pryor NW, Martinez J. 2003. A C-terminal aldehyde insect kinin analog enhances inhibition of weight gain and induces significant mortality in *Helicoverpa zea* larvae. *Peptides* 24:1615–1621.
- Nachman RJ, Kaczmarek K, Williams HJ, Coast GM, Zabrocki J. 2004. An active insect kinin analog with 4-amino-pyroglutamate, a novel cis-peptide bond, type VI β -turn motif. *Biopolymers* 75:412–419.
- Nassel DR. 1996. Neuropeptides in the nervous system of *Drosophila* and other insects: multiple roles as neuromodulators and neurohormones. *Prog Neurobiol* 48:325–420.

- Pietrantonio PV, Gibson GE, Strey AA, Petzel D, Hayes TK. 2000. Characterization of a leucokinin binding protein in *Aedes aegypti* (Diptera: Culicidae) Malpighian tubule. *Insect Biochem Mol Biol* 30:1147–1159.
- Pietrantonio PV, Jagge C, Taneja-Bageshwar S, Nachman RJ, Barhoumi R. 2005. The mosquito *Aedes aegypti* (L.) leucokinin receptor is a multiligand receptor for the three *Aedes* kinins. *Insect Mol Biol* 14:55–67.
- Radford JC, Davies SA and Dow JA. 2002. Systematic GPCR analysis in *Drosophila melanogaster* identifies a leucokinin receptor with novel roles. *J Biol Chem* 277:38810–38817.
- Riehle MA, Garczynski SE, Crim JW, Hill CA, Brown MR. 2002. Neuropeptides and peptide hormones in *Anopheles gambiae*. *Science* 298:172–175.
- Roberts VA, Nachman RJ, Coast GM, Hariharan M, Chung JS, Holman GM, Williams H, Tainer JA. 1997. Consensus chemistry and beta-turn conformation of the active core of the insect kinin neuropeptide family. *Chem Biol* 4:105–117.
- Stables J, Green A, Marshall F, Fraser N, Knight E, Sautel M, Milligan G, Lee M, Rees S. 1997. A bioluminescent assay for agonist activity at potentially any G-protein coupled receptor. *Anal Biochem* 252:115–126.
- Staubli F, Jørgensen TJD, Cazzamali G, Williamson M, Lenz C, Søndergaard L, Roepstorff P, Grimmelikhuijzen CJP. 2002. Molecular identification of the insect adipokinetic hormone receptors. *Proc Natl Acad Sci USA* 99: 3446–3451.
- Torfs P, Nieto J, Veelaert D, Boon D, van de Water G, Waelkens E, Derua R, Calderon J, de Loof A, Schoofs L. 1999. The kinin peptide family in invertebrates. *Ann NY Acad Sci* 897:361–373.
- Veenstra JA, Pattillo JM, Petzel, DH. 1997. A single cDNA encodes all three *Aedes* leucokininins, which stimulate both fluid secretion by the Malpighian tubules and hindgut contractions. *J Biol Chem* 272:10402–10407.